Genetic Backgrounds but Not Sizes of Atherosclerotic Lesions Determine Medial Destruction in the Aortic Root of Apolipoprotein E–Deficient Mice

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Objective—Destruction of the elastic media is the most striking histologic feature of atherosclerotic aortic aneurysms. Apolipoprotein E–deficient (apoE–/–) mice fed a Western diet develop advanced atherosclerotic lesions in the aorta. We sought to assess the integrity of atherosclerotic aortic walls in 2 apoE–/– strains, C57BL/6 (B6) and C3H/HeJ (C3H) that differ markedly in atherosclerosis susceptibility.

Methods and Results—C3H.apoE–/– mice developed much smaller atherosclerotic lesions than did B6.apoE–/– mice after being fed a Western diet for 16 weeks, but the C3H.apoE–/– mice exhibited destruction of the elastic media, including erosion, fragmentation, and focal dilatation beneath plaques. Gelatin and casein zymography showed proteolytic activity of matrix metalloproteinases (MMPs) –9, –2, and –12 in aortic tissues and of MMP-9 and –12 in macrophages from both strains. However, C3H.apoE–/– mice showed significantly increased MMP-2 and –12 activity in aortas and macrophages compared with those from B6.apoE–/– mice. MMP-9 activity was comparable in aortic tissues of the 2 strains, but it was significantly higher in macrophages from C3H.apoE–/– than from B6.apoE–/– mice.

Conclusions—Data indicate that genetic backgrounds but not sizes of atherosclerotic lesions determine medial destruction in the aortic root of apoE–/– mice and that an increase in MMP proteolytic activity might contribute to the medial destruction of aortic walls in C3H.apoE–/– mice. (Arterioscler Thromb Vasc Biol. 2003;23:1901-1906.)

Key Words: atherosclerosis ■ genetic predisposition ■ aortic aneurysms ■ matrix metalloproteinases

Atherosclerosis is a chronic inflammatory disease of large and medium arteries characterized by lipid deposition in the arterial wall.1,2 This disease progresses from foam cell lesions to advanced lesions with fibrous caps and necrotic lipid cores. Rupture of unstable plaques results in the formation of occluding thrombi, which trigger complications such as heart attack and stroke. On the other hand, inflammatory cells such as macrophages and lymphocytes in atherosclerotic lesions might infiltrate into the media and adventitia and impair the connective tissue, especially the elastic lamellae.3,4 The vessel wall is then unable to withstand the expansive force of blood pressure, and aneurysms ensue.

Aortic aneurysms are a common complication consequent to advanced atherosclerotic lesions in elderly individuals.3 The most characteristic histologic change of atherosclerotic aortic aneurysms is the destruction of medial elastic lamellae.3,4 In addition, medial and adventitial infiltration by macrophages and lymphocytes and loss of smooth muscle cells are striking in atherosclerotic aortic aneurysms in humans. Degeneration of the elastic media has been attributed to proteolytic degradation of structural proteins by proteases released by inflammatory cells. The best-studied group of such enzymes involved is the matrix metalloproteinases (MMPs), a family of enzymes that share the ability to degrade many molecules of the extracellular matrix. MMPs are released into tissues as inactive zymogens that can be activated by plasmin and other activators. The activity of MMPs is inhibited by the endogenous tissue inhibitors of metalloproteinases-1 through -4. Increased expression of MMP-1, -2, -3, -9, and -12 has been observed in aneurysm walls.5–8 Targeted gene disruption of MMP-2, -3, and -9 suppresses the development of experimental abdominal aortic aneurysms.9–11 The absence of tissue inhibitor of metalloproteinase-1 enhances MMP activity and promotes aneurysm formation.12

Apolipoprotein E–deficient (apoE–/–) mice have hyperlipidemia and develop all phases of atherosclerotic lesions seen in humans.13,14 Carmeliet et al15 reported that at the advanced
lesion stage, apoE<sup>−/−</sup> mice develop atherosclerosis-associated aneurysms in the aorta. However, subsequent studies failed to find evidence of aortic aneurysm formation in the mice. The reasons for the conflicting results are unknown. In the present study, we have provided experimental evidence that genetic backgrounds influence medial destruction beneath atherosclerotic lesions in apoE<sup>−/−</sup> mouse strains. Our results also suggest that elevation in MMP activity in aortas and macrophages might contribute to the medial destruction in C3H<sub>2</sub>.apoE<sup>−/−</sup> mice.

**Methods**

**Mice and Protocols**

B6.apoE<sup>−/−</sup> mice, which had been sequentially backcrossed with C57BL/6J mice for 10 generations, were purchased from the Jackson Laboratories (Bar Harbor, Me). C3H<sub>2</sub>.apoE<sup>−/−</sup> mice were generated in our laboratory by initially crossing B6.apoE<sup>−/−</sup> mice with C3H/HJ<sub>M</sub> mice. The resulting heterozygous apoE± mice were sequentially backcrossed to C3H mice for at least 4 generations, followed by brother-sister mating to generate homozygous apoE<sup>−/−</sup> mice. The mice were raised on a standard rodent chow containing 4% fat (Ralston-Purina Co). At 8 weeks of age, mice (approximately half male and half female) were started on an advanced, Western-type diet containing 42% fat, 0.15% cholesterol, and 19.5% casein without sodium cholate (TD 88137) and maintained on this diet for 16 weeks. All procedures were in accordance with current National Institutes of Health guidelines and approved by the University Animal Care and Use Committees.

**Assessment of Atherosclerotic Lesions**

The method for assessment of atheromatous lesions in the aorta was performed as previously reported by Qiao et al. In brief, animals were killed by cervical dislocation, and the heart and proximal aorta were excised and washed in phosphate-buffered saline. The basal portion of the heart and proximal aorta were embedded in mounting medium (OCT compound [Miles, Inc]), frozen on dry ice, and then sectioned. Sections from the middle portion of the ventricles to the aortic arch were collected on poly-D-lysine–coated slides. In the region from the appearance to the disappearance of the aortic valves, every other section was collected. In all other regions, every fifth section was collected. The total number of sections examined for lesions ranged from 75 to 110 per mouse. Sections were stained with oil red O and hematoxylin, counterstained with fast green, and examined by light microscopy. For the en face assessment of aortic lesions, the aorta containing the ascending, arch, thoracic, and abdominal segments was dissected; gently cleaned of the adventitia; stained with Sudan IV; and imaged with commercially available software (Image-Pro Plus, Media Cybernetics).

To examine structural changes in detail, the aortas of mice were perfusion-fixed in situ by infusion at 80 mm Hg with 10% formalin, and then stored at −70°C until being sectioned. Serial 10-μm-thick cryosections from the middle portion of the ventricles to the aortic arch were collected on poly-D-lysine–coated slides. In the region from the appearance to the disappearance of the aortic valves, every other section was collected. In all other regions, every fifth section was collected. The total number of sections examined for lesions ranged from 75 to 110 per mouse. Sections were stained with oil red O and hematoxylin, counterstained with fast green, and examined by light microscopy. For the en face assessment of aortic lesions, the aorta containing the ascending, arch, thoracic, and abdominal segments was dissected; gently cleaned of the adventitia; stained with Sudan IV; and imaged with commercially available software (Image-Pro Plus, Media Cybernetics).

To examine structural changes in detail, the aortas of mice were perfusion-fixed in situ by infusion at 80 mm Hg with 10% formalin through the left ventricle for 10 minutes. The basal portions of the heart and proximal aorta were then dissected, processed by standard histologic techniques, and embedded in paraffin. Serial 10-μm-thick sections were cut and stained for elastin with van Gieson stain (Sigma).

**Gelatin and Casein Zymography**

The activity of MMPs in aortic tissues and peritoneal macrophages was determined by gelatin and casein zymography. Six-week-old male mice were used for preparation of aortic proteins and macrophage cellular proteins. At this age, apoE<sup>−/−</sup> mice are known to have no detectable atherosclerotic lesions in the aorta. The aortas were washed thoroughly with phosphate-buffered saline containing 5 U/mL heparin through the left ventricle of the heart, cleaned of periadventitial fat and connective tissue, and snap-frozen in LN<sub>2</sub>. The frozen aortas were mechanically broken up, dispersed in a sample buffer (Invitrogen), and centrifuged at 500g for 10 minutes at 4°C; the supernatant was then collected and used for zymography. For isolation of macrophages, mice were injected intraperitoneally with 1 mL of 3% thioglycolate. Five days later, peritoneal macrophages were harvested by lavage of the peritoneal cavity with 40 mL cold phosphate-buffered saline. Red blood cells were removed by lysis with NH<sub>4</sub>Cl (150 mmol/L, pH 7.3). The remaining cells were suspended in the sample buffer and lysed by the freeze-thaw method. Ten micrograms of aortic or macrophage proteins was separated by electrophoresis on 10% gelatin or 12% casein zymogram gels (Invitrogen). The gels were subsequently incubated overnight at 37°C in a buffer provided by the manufacturer. Enzymatic activities were visualized as negative staining with Coomassie blue R-250 and quantified with a densitometer (Molecular Dynamics).

**In Situ Zymography**

In situ zymography was performed to localize gelatinolytic and caseinolytic activity in aortic tissues, as described by Faia et al. Cyanosides (10 μm thick) of the proximal aorta from 12-month-old apoE<sup>−/−</sup> mice were overlaid on 0.5% low-melting-point agarose (Gibco) gels containing the assay solution (50 mmol/L Tris-HCl, 5 mmol/L CaCl<sub>2</sub>, 5 μmol/L ZnCl<sub>2</sub>, pH 7.5) and fluorescein-conjugated gelatin (50 μg/mL, G-1387; Molecular Probes) or casein (100 μg/mL, C-2990) and incubated overnight at 37°C. EDTA (10 mmol/L) was added to the assay solution as controls. Sections were then examined for fluorescence intensity with an epifluorescence microscope (Nikon TE300).

**Plasma Lipid Measurements**

Mice were fasted overnight before blood was collected from retroorbital veins under isoflurane anesthesia. Plasma total cholesterol, HDL cholesterol, and triglycerides were measured by enzymatic assays, as previously described by Hedrick et al.

**Statistical Analysis**

Data are presented as mean±SE, with n indicating the number of mice. Student’s t test was used to determine differences between strains B6 and C3H in lesion formation, MMP activities, and plasma lipid levels. Differences in phenotype frequencies between the 2 strains were tested by χ² analyses. Differences were considered statistically significant at P<0.05.

**Results**

**Atherosclerotic Lesions and Aortic Wall Destruction**

After being fed the Western diet for 16 weeks, B6.apoE<sup>−/−</sup> mice developed diffuse atherosclerotic lesions throughout the aorta. In contrast, atherosclerotic lesions of C3H.apoE<sup>−/−</sup> mice were primarily localized to the aortic root and arch (Figure 1). The size of the lesions in the aortic root was quantified after transverse cryosections were stained with oil red O. B6.apoE<sup>−/−</sup> mice (n=15) developed much larger atherosclerotic lesions than their C3H counterparts (n=10; 601 800±40 200 vs 63 000±5800 μm² per cross section per mouse; P<0.0001; Table 1). Although C3H.apoE<sup>−/−</sup> mice developed smaller aortic lesions, the media of their aortic walls showed erosion, disruption, or fragmentation by atherosclerotic lesions (Figure 2A and Table 1). Some plaques completely perforated the medial layer and protruded into the adventitia (Figure 2B). Focal dilatation of the medial layer was observed in 5 of 10 mice (Figure 2C). Calcium deposits were also observed in the media that was impaired by atherosclerotic lesions (6 of 10 mice; Figure 2D). In contrast, in B6.apoE<sup>−/−</sup> mice (n=15), we did not observe erosion, disruption, or fragmentation of the media by adjacent athero-
sclerotic lesions, even though these mice had developed much larger lesions. In addition, in both strains, the aortic walls without atherosclerotic lesions did not show any signs of medial destruction.

To confirm the aforementioned findings, a separate set of experiments was performed in which the aortic roots of B6.apoE−/− (n = 5) and C3H.apoE−/− mice (n = 10) were processed by standard histologic techniques and stained with elastin–van Gieson’s stain. As shown in Figure 3, the elastic laminae of the media in B6 mice were continuous and did not show signs of impairment by atherosclerotic lesions. In contrast, atherosclerotic lesions in C3H mice infiltrated into the media of the aortic wall and degraded the elastic laminae in an internal-to-external gradient. The elastic laminae exhibited fragmentation or rupture of elastic layers. Autofluorescence analysis of endogenous elastin revealed that the elastic laminae were eroded or disrupted by atherosclerotic lesions (Figure 3C and 3D).

Activity of MMPs
Aortic proteins and macrophage cellular proteins prepared from 6-week-old male B6.apoE−/− and C3H.apoE−/− mice were analyzed by gelatin and casein zymography (Figure 4A). In aortic tissues, the predominant gelatinolytic bands occurred at 92, 72, and 62 kDa in both strains, corresponding to MMP-9 and pro–MMP-2 and the active form of MMP-2, respectively. In macrophages, the predominant gelatinolytic band occurred at 92 kDa in both strains, corresponding to MMP-9. In both aortic tissues and macrophages, the predominant caseinolytic band occurred at 22 kDa, corresponding to the active form of MMP-12. In both aortic tissues and macrophages, C3H.apoE−/−
mice showed significantly increased activity levels of pro-MMP-2, MMP-2, and MMP-12 when compared with those of B6.apoE<sup>-/-</sup> mice (P=0.00136 to 0.03; Figure 4B). In aortic tissues, MMP-9 activity was comparable between the 2 strains (P=0.95), but in macrophages, its activity was significantly higher in C3H.apoE<sup>-/-</sup> mice than in B6.apoE<sup>-/-</sup> mice (P=0.030).

To determine the cell types that express MMPs in the aortic wall, in situ zymography with fluorescent gelatin and casein was performed on the aortas of 12-month-old apoE<sup>-/-</sup> mice that had been fed a chow diet (Figure 4C). As shown in A through D, strong gelatinolytic activity was detected in atherosclerotic lesions, especially at the shoulder of the lesions. Mild gelatinolytic activity was detected in the media of the arterial wall without atherosclerotic lesions. Enzymatic activity was suppressed by the addition of 10 mmol/L EDTA, indicating that the gelatinolytic activity was derived from MMPs. In situ zymography with casein indicated that caseinolytic activity was pronounced in the media of the aortic wall and at the caps of atherosclerotic lesions (E and G), whereas it was less pronounced at the core of atherosclerotic lesions. The enzymatic activity was mildly inhibited by EDTA (F and H).

### Plasma Lipid Levels

After being fed the Western diet for 16 weeks, both B6.apoE<sup>-/-</sup> and C3H.apoE<sup>-/-</sup> mice developed extreme hypercholesterolemia (Figure 5). The total cholesterol level was 1187±59 mg/dL in B6.apoE<sup>-/-</sup> mice and 1088±40 mg/dL in C3H.apoE<sup>-/-</sup> mice, although the difference was not statistically significant (P=0.10). C3H.apoE<sup>-/-</sup> mice had significantly increased levels of HDL cholesterol (119±11 vs 27±4 mg/dL; P=0.00001) and triglycerides (105±13 vs 29±7 mg/dL; P=0.00014) compared with B6.apoE<sup>-/-</sup> mice.

### Discussion

In the present study, we examined phenotypic differences in the integrity of atherosclerotic aortic walls in 2 apoE<sup>-/-</sup> mouse strains, B6 and C3H, which are known to differ markedly in atherosclerosis susceptibility. The major finding was that C3H.apoE<sup>-/-</sup> mice exhibited atherosclerosis-associated medial destruction, whereas B6.apoE<sup>-/-</sup> mice were resistant to medial destruction, despite the fact that they developed much larger atherosclerotic lesions. Carmeliet and colleagues<sup>15</sup> first reported that apoE<sup>-/-</sup> mice, which were bred on a mixed genetic background of 75% B6 and 25% 129SvJ, developed atherosclerotic aneurysms in the aortas when they were fed an atherogenic diet with cholate for 10 weeks. However, 2 subsequent studies reported that on the B6 genetic background, apoE<sup>-/-</sup> mice were resistant to medial destruction.

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**Figure 4.** A, Gelatinolytic and caseinolytic activity in lysates of aortas and peritoneal macrophages harvested from 6-week-old male B6.apoE<sup>-/-</sup> and C3H.apoE<sup>-/-</sup> mice. Proteins (10 μg) were separated by electrophoresis on 10% gelatin or 12% casein zymogram gels. Each lane represents an individual mouse except for the middle lane, which is the protein standard. Presence of MMP-9, MMP-2, and MMP-12 is indicated by their lytic activity and molecular weight. B, Optical density of MMP-9, pro-MMP-2, MMP-2, and MMP-12 shown in A. Values are mean±SEM for 5 mice. *P<0.05 vs B6.apoE<sup>-/-</sup> mice. C, In situ zymography with fluorescent gelatin and casein in the proximal aortas of 12-month-old B6.apoE<sup>-/-</sup> and C3H.apoE<sup>-/-</sup> mice that were fed a chow diet. Cryosections were overlaid on low-melting-point agarose (0.5%) gels containing the assay solution (50 mmol/L Tris·HCl, 5 mmol/L CaCl<sub>2</sub>, 5 μmol/L ZnCl<sub>2</sub>·pH 7.5) and fluorescein-conjugated gelatin (50 μg/mL) or casein (100 μg/mL) and incubated overnight at 37°C. Gelatinolytic activity was obvious in atherosclerotic lesions and was mild in the median arterial wall (A and C) but inhibited after addition of 10 mmol/L EDTA (B and D). Caseinolytic activity was pronounced in the median arterial wall and at the cap of atherosclerotic lesions (E and G), whereas it was less pronounced at the core of atherosclerotic lesions. Enzymatic activity was mildly inhibited by EDTA (F and H).
ground, apoE−/− mice were resistant to aortic aneurysm forma-
tion.16,17 In those studies, the apoE−/− mice were at least 7
months old or were fed a Western-type diet for >16 weeks.
At these stages, apoE−/− mice should have developed advanced
atherosclerotic lesions in the aorta.19 In the present study, we
also found that B6.apoE−/− mice were resistant to medial
destruction. The discrepancy in the results between the
studies of Carmeliet et al15 and others suggest that cholate-
containing diets and/or genetic backgrounds contribute to
atherosclerotic aneurysm formation in apoE−/− mice. Indeed,
the cholate-containing diet has been shown to cause a chronic
inflammatory state, with the expression of inflammatory and
oxidative stress genes, in the liver and probably also in the
vessel walls.20 Inflammation is known to play a key role in
the development and progression of aortic aneurysms.21
Nevertheless, given the present findings that C3H.apoE−/−
mice developed aortic destruction without being fed the
cholate diet, genetic backgrounds appear to contribute more
significantly to the deterioration of the elastic media in apoE−/−
mice.

The major finding of this study is that genetic backgrounds
but not sizes of atherosclerotic lesions determine medial
destruction in apoE−/− mice. This finding provides an expan-
sion for the puzzling association between atherosclerosis and
atherosclerotic disease. Indeed, although the great
majority of aortic aneurysms are associated with atheroscle-
rosis, many patients suffering from atherosclerotic disease
never develop aortic aneurysms.3 The factors that contribute
to the progression from an intimal lesion (atherosclerosis) to
major medial damage (aneurysms) are not well known.20
However, genetic factors appear to be a major determinant for
the progression leading to aortic aneurysm formation. Indeed,
prospective family studies indicate that male siblings of
individuals affected with aortic aneurysms have an increased
risk of 11% to 32% for developing the disorder compared
with the general population risk of 2% to 5%.25,26

MMP-2, MMP-9, and MMP-12 are able to directly degrade
elastin or fibrillar collagen; we thus investigated their activ-
ities in aortic tissues and macrophages from the 2 apoE−/−
strains. A notable finding of this study is the obvious activity
of all 3 MMPs detected in aortas, even though they had no
atherosclerotic lesions. In a recent study, Galis et al27 also
detected apparent MMP-9 and MMP-2 activity in the carotid
arteries of mice. MMP-12 is known to be produced by
monocytes/macrophages,28,29 aortic intimal smooth muscle
cells,30 and cultured medial smooth muscle cells that lose
contractility.29 In this study, we found that MMP-12 was also
produced by normal aortas of mice. Interestingly, although
the activity of MMP-9 in the aorta was comparable between
the 2 strains, C3H mice exhibited significantly increased
proteolytic activity of MMP-2 and MMP-12 when compared
with B6 mice. Given that MMP-2 and MMP-12 have potent
elastolytic ability, it is plausible to speculate that these 2
MMPs produced by arterial wall cells played a role in the
destruction of the elastic media in C3H mice.

In this study, we found that medial destruction was
associated with infiltration of the arterial walls by atheroscle-
rotic lesions in C3H.apoE−/− mice (Figures 2 and 3). Thus, the
increase in activity of MMP-9 and MMP-12 in macrophages
observed in this study could contribute significantly to the
destruction of the media in C3H mice. In macrophages, we
found that MMP-2 activity was not detectable in B6 mice and
was very limited in C3H mice. This finding is consistent with
the immunohistochemical result of Davis et al,31 who re-
ported that MMP-2 is expressed by smooth muscle cells and
fibroblasts but rarely expressed by macrophages. These
observations suggest that MMP-2 produced by macrophages
contributed less significantly to the medial destruction in
C3H mice.

Hypercholesterolemia has been shown to increase mono-
cyte infiltration in injured aortic walls and promote aortic
aneurysm formation in rabbits.32,33 However, in our experi-
ments, we found that the 2 apoE−/− strains had comparable
plasma levels of total cholesterol. Thus, hypercholesterolemia
is unlikely to explain the difference between them in medial
destruction. C3H.apoE−/− mice had a dramatically increased
HDL cholesterol level in comparison with B6.apoE−/− mice.
HDL is known to inhibit monocyte infiltration and alleviate
inflammation; thus, the increased HDL cholesterol level
could not explain the medial destruction of C3H mice.

Studies of the mechanisms responsible for aneurysm for-
mation and progression have been hampered by the lack of
availability of animal models. Carrell et al34 maintained that
an ideal model of aortic aneurysms should include all of the
pathologic features observed in the human condition, such as
atherosclerosis, disruption of elastic lamellae in the tunica
media, and adventitial inflammation. However, few animal
models reproduce all of these features. In rabbits, hyperlip-
idemia results in extensive aortic atherosclerosis but does not
induce aortic aneurysms. To induce aneurysms, CaCl2 and
thioglycolate have to be applied to the adventitia to enhance
aortic wall inflammation.34 Aortic aneurysms can be induced
in B6.apoE−/− mice by angiotensin infusion, but in this model,
aneurysms are independent of atherosclerotic lesions.16 In
contrast, C3H.apoE−/− mice develop spontaneous atheroscle-
rosis and display disruption of the elastic lamellae and infiltration by macrophages, thus providing an experimental tool to study atherosclerotic aortic aneurysms. However, it is worth noting that although C3HapoE−/− mice exhibited obvious medial destruction, there was no gross aneurysmal dilatation of the aortic walls. The dissociation between medial destruction and aneurysmal dilatation might be explained by the finding that advanced atherosclerotic lesions of apoE−/− mice contain a large fraction of fibrotic tissues as well as calcification,13,19 which are known to limit dilatation of the vessel wall.

In summary, we have observed the experimental evidence that genetic factors influence atherosclerotic medial wall destruction, at least partially, by modulating the activity of MMPs. Also, these genetic factors seem to be independent of factors that influence the size of atherosclerotic lesions.

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